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FREEZING DAMAGE PROTECTING AGENT AND FREEZING STORAGE METHOD

[凍害保護剤および凍結保存方法]

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(57) [Abstract]

[Constitution]

After soaking microorganism or cell in freezing damage protecting agent for the microorganism or cell which designates that it contains inulin type fructan as active ingredient as feature and in solution which includes the inulin type fructan, freezing or lyophilizing freezing storage method. of microorganism or the cell which designates that it does as feature

[Effect (s)]

If it uses freezing damage protecting agent of this invention, when freezing doing cell of microorganism and plant, animal, you can control freezing damage, the survival rate can improve.

Therefore, as for this invention usefulness is high in food industry, drug industry or other field.

[Claim (s)]

[Claim 1]

Freezing damage protecting agent. for microorganism or cell which designates that it contains inulin type fructan as active ingredient as feature

[Claim 2]

Freezing damage protecting agent. which is stated in Claim 1 where inulin type fructan is mixture of degree of polymerization 3 - 6

[Claim 3]

Freezing damage protecting agent. which is stated in Claim 1 where inulin type fructan is any of degree of polymerization 3, 4 and 5

[Claim 4]

Freezing damage protecting agent. which is stated in Claim 1 where microorganism or cell is cultured cell of Bifidobacteria, acidophilus, E. coli, Bacillus subtilis, yeast or plant, animal

[Claim 5]

After soaking microorganism or cell in solution which includes the inulin type fructan, freezing or lyophilizing freezing storage method. of microorganism or the cell which designates that it does as feature

[Claim 6]

freezing storage method. which is stated in Claim 5 where inulin type fructan is degree of polymerization 3 - 6

[Claim 7]

freezing storage method. which is stated in Claim 5 where inulin type fructan is any of degree of polymerization 3, 4 and 5

[Claim 8]

freezing storage method. which is stated in Claim 5 where microorganism or cell is cultured cell of Bifidobacteria, acidophilus, E. coli, Bacillus subtilis, yeast or plant, animal

[Description of the Invention]

[0001]

[Field of Industrial Application]

this invention regards freezing damage protecting agent and freezing storage method which designate inulin type fructan as active ingredient.

Furthermore in detail, when freezing doing cultured cell of Bifidobacteria, acidophilus, E. coli or other microorganism or plant, animal, you control freezing damage which happens, survival rate you regard freezing damage protecting agent and freezing storage method which improve.

[0002]

[Prior Art And Problems To Be Solved By The Invention]

Storage & preservation of microorganism and cell are used for the preservation of system which is superior simply, furthermore, the use limit is expanding.

With for example microorganism, as for Bifidobacteria and lactic acid bacteria, etc., for complexity it is done as foodstuff material where lyophilized cell mass has integral intestinal effect.

In addition, E. coli or other preservation which specific gene is introduced has become important problem on genetic engineering.

[0003]

Generally, freezing preservation is done for long-term storage and preservation of microorganism and cell or other body liquid.

Until recently, after in physiological diluting with acceptable freezing damage protecting agent at time of freezing preserving a this way, method which freezing it stores & preserves is majority.

But, as for freezing being severe for organism, there is many a thing which survival rate decreases because of thermal impact and crystal production which it occurs with freezing and thawing step.

Therefore, freezing damage protecting agent and development of freezing storage method which raise from survival rate after freezing preserving were desired.

[0004]

Polge, Nature (Nature), 164 volumes, development of protecting agent in order 666 page, 1949]. after that to control freezing damage is done. In lyophilizing formulating of, for example, Bifidobacteria and lactic acid bacteria, survival rate as protecting agent which is added in order to improve lactulose (Japan Unexamined Patent Publication Showa 52-151787 disclosure), vitamin E (Japan Examined Patent Publication Sho 53-5747 disclosure), corn steep liquor (Japan Unexamined Patent Publication Showa 58-104787 disclosure), the raw starch (Japan Unexamined Patent Publication Showa 58-149675 disclosure) and method which uses cyclodextrin (Japan Unexamined Patent Publication Showa 63-251080 disclosure) is known. But, these method, said protecting agent is used for large scale, substance

being the expensive, had or other problem where tastiness is not desirable. In addition, when freezing it preserves animal cell, addition of the blood serum is needed in freezing culture medium, or also method which methylcellulose and trehalose (Japanese Publication of International Patent Application 4-501112 disclosure) or adds trehalose and gelatin (Japan Unexamined Patent Publication Hei 5-7489 disclosure) in place of blood serum as protecting agent, is established. But, these method improvement of survival rate has become problem. In order to control freezing damage of microorganism and cell, the method which in physiological is diluted with acceptable protecting agent is examined, that glycerol is effective, it is discovered

[0005]

[Means to Solve the Problems]

Then as for these inventors in order to control extermination of microorganism and cell in freezing process fact that extermination of microorganism and the cell can be controlled considerably various result and inulin type fructan which were examined as freezing damage protecting agent in diluent by using, in time of freezing preservation or time of lyophilizing was discovered, this invention was completed.

[0006]

this invention after soaking microorganism or cell in freezing damage protecting agent for microorganism or cell which designates that it contains the inulin type fructan as active ingredient as feature and in solution which includes inulin type fructan, freezing or is something which offers freezing storage method of microorganism or cell which designates that lyophilizing it does as feature.

[0007]

There are various ones as microorganism where this invention is applied, the for example Bifidobacterium adolescentis, Bifidobacterium infantis, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium breve or other Bifidobacteria, Streptococcus faecalis, Lactobacillus acidophilus or other lactic acid bacteria, Escheria coli K-12 like coli, Bacillus subtilis Marburg 168 strain they can list yeast like the Bacillus subtilis, Saccharomyces cerevisiae like.

[0008]

In addition, you can list human, cattle, horse and cultured cell, etc., of the embryo, cancer cell, T-cell leukemia cell, hybridoma, fibroblast, blood vessel configuration cell, bone marrow cell, dispersed islets of Langerhans cell, fish of goat, sheep, rabbit, hamster, rat, mouse as animal cell.

As plant cell, rice, wheat, Catharanthus roseus G. Don, Zea mays L. (Corn), Saccharum officinarum L. (sugar cane), tobacco, lavender, Malus pumila Miller var. domestica Schneider (apple), Daucus carota L. var. sativa DC. (carrot), soybeans, Marchantia polymorpha, strawberry, Solanum tuberosum L. (potato), Dianthus spp. (carnation), Pisum sativum L. (garden pea), Asparagus officinalis L. (asparagus), Brussels sprout, pear or other cultured cell and protoplast, shoot apex and uncertain embryo, etc., can be listed.

[0009]

microbe collection it does microorganism culture after doing, with centrifugal separation, etc., with culture medium of option, wet cell mass which washed with suitable washing liquid is utilized, cell can use cell which culture is done with the method of shaking culture or other option.

It uses microorganism or cell which it acquires, that way or as the suspension which adds buffer of trace, suspension it does in the diluent which includes inulin type fructan which disinfection is done beforehand.

[0010]

With alone, or appropriateness combination and according to need or adding skim milk, dimethyl sulfoxide, glycerin, fructose, sucrose, lactose, vitamin, etc., to additional simultaneously as other component as the active ingredient including inulin type fructan, you can use component of the diluent.

[0011]

You should have used concentration of inulin type fructan which is used for this invention, with concentration which is suited for microorganism and the cell which if freezing are done, in range of preferably 1 - 40 percent by weight uses.

In addition, you can use inulin type fructan of this invention, as the one part or all of composition of freezing damage

protecting agent.

inulin type fructan with fructan of degree of polymerization 3 or greater which fructose has connected to sucrose straight chain, usually those of degree of polymerization 3 - 15, preferably degree of polymerization 3 - 6 being the alone, or is used as mixture of 2 kinds or more.

[0012]

These inulin type fructan or mixture chicory and by extracting from the Helianthus tuberosus L., etc., or can acquire enzyme which has fructose metastasis power in sucrose by operating.

When exemplary inulin type fructan is illustrated, you can list those below, but this invention is not something which is limited in these.

inulin type fructan which is acquired by extraction of for example Helianthus tuberosus L., those of degree of polymerization 3 - 6 (30 - 50 percent by weight), those of degree of polymerization 7 or greater (20 - 50 percent by weight), has sugar composition of the monosaccharide and biose (10 - 30 percent by weight), enzyme of Aspergillus niger derivation operating, the inulin type fructan (trade name: Meioligo G; Meiji Seika Kaisha, Ltd. (DB 69-054-1941) supplied) which is acquired those of degree of polymerization 3 - 6 (55 - 60 percent by weight), has the sugar composition of monosaccharide and biose (40 - 45 percent by weight) in sucrose.

Furthermore, above-mentioned mixture making use of column chromatography or the film, etc., fructan mixture which designates sugar of degree of polymerization 3 - 6 as the main component portion by refining can be acquired.

for example above-mentioned Meioligo G making use of column chromatography be able to acquire inulin type fructan (trade name: Meioligo P) which contains those of degree of polymerization 3 - 6 at ratio of 95 percent by weight or more portion by refining, inulin type fructan of the degree of polymerization 3 - 6 (70 percent by weight or more) can be acquired even from Helianthus tuberosus L. extract.

Furthermore, inulin type fructan, for example degree of polymerization 4 which designates single degree of polymerization as the main component due to especially combining

column chromatography and crystallization, etc., (nistose), the degree of polymerization 5 (fructocyl-nistose) can be acquired.

[0013]

this invention freezing damage protecting agent for microorganism or cell which designates that it contains inulin type fructan as active ingredient as feature after freezing preserving has shown large improvement of the ratio (survival rate) where microorganism and cell survive in comparison with the known freezing damage protecting agent which includes active ingredient which is known until recently, extermination in freezing process of microorganism or cell can be prevented effectively.

[0014]

[Working Example (s)]

Next, Working Example of this invention is shown, but this is not something wherein illustration, this invention is limited to last in this.

Working Example 1

Bifidobacteria (Bifidobacterium longum) 37 deg C, 24 time anaerobic culture was done with BL culture medium, cell mass was separated from culture fluid after culture due to centrifugal separation at once.

cell mass which microbe collection is done was washed with anaerobic phosphate buffer (pH 7.0), this was done centrifugal separation, microbe collection again.

equivalent 3 min it did wet cell mass which it acquires, offered to experiment below.

(a) nistose (inulin type fructan of degree of polymerization 4) in diluent which includes only 6.7 percent by weight in uniform after suspension, freezing doing wet cell mass which is acquired with - 25 deg C, after 24 hours preserving, thrice it repeated the freezing and thawing (With -25 deg C with freezing, 30 deg C rapidity thawing).

As a result, as for living bacteria count of freezing preservation microbe with 2.08 X 10^8/ml, as for survival rate it was 41.6%.

[0015]

From (b) Helianthus tuberosus L. inulin type fructan which is extracted (Those 52% of composition: degree of polymerization 3 - 10, those 34% above degree of polymerization 11) in the diluent which includes only 6.7 percent by weight wet cell mass after and description above suspension (a) with it operated in same way in uniform.

As a result, as for living bacteria count of freezing preservation microbe with 1.25 X 10^8/ml, as for survival rate it was 25.5%.

In diluent which includes only sucrose 6.7 percent by weight (mole concentration 0.1 M) as (c) Contrasting Example the wet cell mass in uniform after and description above suspension (a) with when it operated in same way, as for living bacteria count of freezing preservation microbe with 6.30 X 10^7/ml, as for survival rate it was 12.9%.

[0016]

Working Example 2

Bifidobacteria (Bifidobacterium adolescentis) 37 deg C, 24 time anaerobic culture was done with BL culture medium, cell mass was separated from culture fluid after culture due to centrifugal separation at once.

cell mass which microbe collection is done was washed with anaerobic phosphate buffer (pH 7.0), this was done centrifugal separation, microbe collection again.

equivalent 4 min it did wet cell mass which it acquires, offered to experiment below.

(a) nistose (inulin type fructan of degree of polymerization 4) it mixed wet cell mass which is acquired, to 3 percent by weight and the diluent which includes skim milk 10 percent by weight with weight ratio 1:1, adjusted pH 7.0 (5 rule sodium hydroxide solution).

aliquot it did mixed solution in Petri dish, lyophilizing did with - 20 deg C.

As for living bacteria count after lyophilizing with 1.18 X

10^{11}/g, as for survival rate it was 33.5%.

[0017]

Meioligo P which is a mixture of (b) inulin type fructan (Those 44.4% of trade name, Meiji Seika Kaisha Ltd. (DB 69-054-1941) supplied, composition: degree of polymerization 3, those 42.9% of degree of polymerization 4, those 8.9% of degree of polymerization 5, those 0.6% of degree of polymerization 6) after mixing 3 percent by weight and diluent and wet cell mass which include skim milk 10 percent by weight with weight ratio 1:1, description above (a) with it operated in same way.

As for living bacteria count after lyophilizing with 1.08 X 10^{11}/g, as for survival rate it was 30.6%.

(c) fructocyl-nistose (inulin type fructan of degree of polymerization 5) After mixing 3 percent by weight and diluent and wet cell mass which include skim milk 10 percent by weight with weight ratio 1:1, description above (a) with when it operated in same way, as for living bacteria count after lyophilizing with 1.09 X 10^{11}/g, as for survival rate it was 30.8%.

After mixing wet cell mass to diluent which includes lactose 3 percent by weight and the skim milk 10 percent by weight as (d) Contrasting Example with weight ratio 1:1, description above (a) with it operated in same way.

As a result, as for living bacteria count after lyophilizing with 5.88 X 10^{10}/g, as for survival rate it was 16.7%.

[0018]

Working Example 3

E. coli (Escheria coli K-12) 37 deg C, 24 time culture was done with L broth, cell mass was separated from culture fluid after culture due to centrifugal separation at once.

cell mass which microbe collection is done was washed with phosphate buffer (pH 7.0), this was done centrifugal separation, microbe collection again.

equivalent 3 min it did wet cell mass which it acquires, offered to experiment below.

(a) nistose (inulin type fructan of degree of polymerization 4) in diluent which includes only 10 percent by weight suspension it did wet cell mass which is acquired in uniform, freezing did with - 25 deg C.

After 24 hours preserving, freezing and thawing (With -25 deg C with freezing, 30 deg C rapidity thawing) thrice was repeated.

As a result, as for living bacteria count of freezing preservation microbe as for the survival rate it was 41.6% with 3.99 X 10^8/ml.

[0019]

(b) 1 - kestose (inulin type fructan of degree of polymerization 3) In diluent which includes only 10 percent by weight suspension doing wet cell mass in uniform, description above (a) with when it operated in same way, as for living bacteria count of freezing preservation microbe with 2.47 X 10^8/ml, as for survival rate it was 25.8%.

In diluent which includes only trehalose 10 percent by weight as (c) Contrasting Example the suspension doing wet cell mass in uniform, description above (a) with when it operated in same way, as for living bacteria count of freezing preservation microbe as for survival rate it was 20.2% with 1.86 X 10^6/ml.

In diluent which includes only sucrose 10 percent by weight as (d) Contrasting Example the suspension doing wet cell mass in uniform, description above (a) with it operated in same way.

As a result, as for freezing preservation microbe as for survival rate it was18.3% with living bacteria count 1.77X 10^8/ml.

[0020]

Working Example 4

lactic acid bacteria (Lactobacillus acidophilus) 37 deg C, 24 time culture was done with ILS culture medium, after the culture cell mass was separated from culture fluid at once with centrifugal separation.

cell mass which microbe collection is done was washed with phosphate buffer (pH 7.0), this was done centrifugal separation, microbe collection again.

equivalent 2 min it did wet cell mass which it acquires, offered to experiment below.

[0021]

nistose (inulin type fructan of degree of polymerization 4) In diluent which includes only 6.7 percent by weight suspension it did(a) wet cell mass in uniform, - freezing did with 25 deg C.

After 24 hours preserving, freezing and thawing (with -25 deg C with freezing, 30 deg C rapidity thawing) thrice was repeated.

As for living bacteria count of freezing preservation microbe as for survival rate it was47.6% with 4.0 X 10^8/ml.

In diluent which includes only sucrose 6.7 percent by weight as (b) Contrasting Example the suspension doing wet cell mass in uniform, description above (a) with it operated in same way.

As a result, as for living bacteria count of freezing preservation microbe as for the survival rate it was 15.7% with 1.32 X 10^8/ml.

[0022]

Working Example 5

With MEM culture medium which adds 10% fetal calf serum making use of Hela cell of cancer cell derivation as cultured cell, with 37 deg C culture after doing, treating with 0.25% trypsin (EC 3.4.21.4) liquid, it peeled cell.

You inserted this in centrifuge tube, 5 - 10 min centrifugation did with 500 - 600 rpm and collected cell.

equivalent 3 min it did cell which it acquires, offered to experiment below.

In order to become final concentration 10 percent by weight, adding (a) nistose (inulin type fructan of degree of polymerization 4) to the multiplication culture medium which includes blood serum agitating well, it manufactured culture medium for freezing.

With cold warm state, suspension it did in culture medium for the freezing including cell and 1 - 2 hours standing did, after

permeating, enclosed freezing damage protecting agent into ampoule in intracellular.

- freezing it did this ampoule with 80 deg C and 9 day preserved.

With constant temperature tank of 37 deg C after thawing, 10 - 20 times extent it diluted with multiplication culture medium, 5 - 10 min centrifugation did with 500 - 600 rpm and collected cell.

cell which it melts verified living and dead making use of trypan blue dye, result 93% survival which calculated survival rate had done.

[0023]

In order to become final concentration 10 percent by weight, adding glycerin to multiplication culture medium which includes blood serum agitating well as (b) Contrasting Example, it manufactured culture medium for freezing.

This description above (a) with when it operated in same way, the survival rate after freezing and thawing was 65%.

In order to become final concentration 10 percent by weight, adding glucose to multiplication culture medium which includes blood serum agitating well as (c) Contrasting Example, it manufactured culture medium for freezing.

This description above (a) with when it operated in same way, the survival rate after freezing and thawing was 62%.

[0024]

Working Example 6

With Ham'sF12culture medium which adds 10% fetal calf serum making use of CHO-K1 cell of Chinese hamster ovary derivation as cultured cell, with 37 deg C culture after doing, treating with 0.25% trypsin (EC 3.4.21.4) liquid, it peeled cell.

You inserted this in centrifuge tube, 5 - 10 min centrifugation did with 500 - 600 rpm and collected cell.

equivalent 3 min it did cell which it acquires and offered to experiment below.

In order to become final concentration 10 percent by weight, adding (a) nistose (inulin type fructan of degree of polymerization 4) to the multiplication culture medium which includes blood serum agitating well, it manufactured culture medium for freezing.

With cold warm state, suspension it made culture medium for the freezing including cell and 1 - 2 hours standing did, after permeating, enclosed freezing damage protecting agent into ampoule in intracellular.

- freezing it did this ampoule with 80 deg C and 9 day preserved.

With constant temperature tank of 37 deg C after thawing, 10 - 20 times extent it diluted with multiplication culture medium, 5 - 10 min centrifugation did with 500 - 600 rpm and collected cell.

cell which it melts verified living and dead making use of trypan blue dye, result and 88% survival which calculated survival rate had done.

[0025]

In order to become final concentration 10 percent by weight, adding glycerin to multiplication culture medium which includes blood serum agitating well as (b) Contrasting Example, it manufactured culture medium for freezing.

This description above (a) with when it operated in same way, the survival rate after freezing and thawing was 61%.

In order to become final concentration 10 percent by weight, adding glucose to multiplication culture medium which includes blood serum agitating well as (c) Contrasting Example, it manufactured culture medium for freezing.

This description above (a) with when it operated in same way, the survival rate after freezing and thawing was 48%.

In order to become final concentration 10 percent by weight, adding dimethyl sulfoxide to multiplication culture medium which includes blood serum agitating well as (d) Contrasting Example, it manufactured culture medium for freezing.

Description above (a) with it operated this in same way.

As a result, survival rate after freezing and thawing was 78%.

[0026]

Working Example 7

culture it did callus of hypocotyl derivation of Daucus carota L. var. sativa DC. (carrot) with liquid culture medium of suitable option, in 0.7 M mannitol treated with Meicelase P-1 (trade name, Meiji Seika Kaisha, Ltd. (DB 69-054-1941) supplied), filtration and centrifugation, washed, acquired protoplast suspension.

equivalent 3 min it did protoplast which it acquires and offered to experiment below.

In order to become final concentration 10%, adding to above-mentioned culture medium agitating well, filtration & sterilization doing final concentration 20 percent by weight, dimethyl sulfoxide, it designated (a) nistose (inulin type fructan of degree of polymerization 4) as culture medium for freezing.

While with cold warm state, at a time trace agitating the culture medium for this freezing calmly in protoplast suspension, it added.

30 minute - 1 hour extent standing it did, after permeating, freezing designated freezing damage protecting agent as inside protoplast with liquid nitrogen medium- 196 deg C and 9 day preserved.

It diluted with liquid culture medium which with constant temperature tank of 37 deg C includes 0.4 M mannitol after rapidity thawing and in ice, centrifugation did and collected protoplast.

protoplast which it melts verified living and dead making use of Evan's blue stain, result 52% survival which calculated survival rate had done.

[0027]

In order to become final concentration 10%, adding final concentration 20 percent by weight, dimethyl sulfoxide to above-

mentioned culture medium agitating well, filtration & sterilization it did glucose as the (b) Contrasting Example, made culture medium for freezing.

This description above (a) with when it operated in same way, the survival rate after freezing and thawing was 42%.

In order to become final concentration 10%, adding final concentration 20 percent by weight, dimethyl sulfoxide to above-mentioned culture medium agitating well, filtration & sterilization it did sucrose as the (c) Contrasting Example, made culture medium for freezing.

This description above (a) with when it operated in same way, the survival rate after freezing and thawing was 28%.

[0028]

[Effects of the Invention]

If freezing damage protecting agent of this invention is used, when freezing doing cell of microorganism and plant, animal, you can control freezing damage, the survival rate can improve.

Therefore, as for this invention usefulness is high in food industry, drug industry or other field.

PATENT ABSTRACTS OF JAPAN

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(54) FREEZING DAMAGE-PROTECTING AGENT AND FREEZING AND STORING METHOD

(57)Abstract:
PURPOSE: To obtain a freezing damage-protecting agent capable of suppressing hindrance due to freeze and improving survival rate in freezing a cell of a microorganism, a plant or an animal and having its high serviceability in fields of food industry, medicine industry, etc.
CONSTITUTION: This freezing damage-protecting agent for microorganisms or cells contains inulin type fructan as an active ingredient. The method for carrying out freeze storage of a microorganism or a cell is to immerse the microorganism or the cell in a solution containing the inulin type fructan and then freeze or lyophilize the microorganism or the cell.

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CLAIMS

[Claim(s)]
[Claim 1] The frost damage protective agent for the microorganism characterized by containing an inulin mold fructan as an active principle, or a cell.
[Claim 2] The frost damage protective agent according to claim 1 whose inulin mold fructan is the mixture of polymerization degree 3-6.
[Claim 3] The frost damage protective agent according to claim 1 whose inulin mold fructan is either of the polymerization degree 3, 4, and 5.
[Claim 4] The frost damage protective agent according to claim 1 a microorganism or whose cell is a cultured cell of lactobacillus bifidus, lactic acid bacteria, Escherichia coli, a Bacillus subtilis, yeast or vegetation, and an animal.
[Claim 5] The microorganism characterized by making it freeze or freeze-dry after dipping a microorganism or a cell into the solution containing an inulin mold fructan, or the cryopreservation approach of a cell.
[Claim 6] The cryopreservation approach according to claim 5 that inulin mold fructans are polymerization degree 3-6.
[Claim 7] The cryopreservation approach according to claim 5 that an inulin mold fructan is either of the polymerization degree 3, 4, and 5.
[Claim 8] The cryopreservation approach according to claim 5 that a microorganism or a cell is a cultured cell of lactobacillus bifidus, lactic acid bacteria, Escherichia coli, a Bacillus subtilis, yeast or vegetation, and an animal.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to the frost damage protective agent and the cryopreservation approach of making an inulin mold fructan an active principle. Furthermore, the freezing failure encountered in it when freezing the cultured cell of microorganisms, such as lactobacillus bifidus, lactic acid bacteria, and Escherichia coli, or vegetation, and an animal in a detail is controlled, and it is related with the frost damage protective agent and the cryopreservation approach of raising a survival rate.
[0002]
[Description of the Prior Art] Storage and preservation of a microorganism or a cell are not only used for preservation of the network which was only excellent, but the use range is expanding it. For example, by the microorganism, pressure of business of lactobacillus bifidus, the lactic acid bacteria, etc. is carried out as a food material in which the lyophilized cell has the ready intestines effectiveness. Moreover, preservation of the Escherichia coli introduced in the specific gene has been an important technical problem on gene engineering.

[0003] Generally, cryopreservation is performed for long term storage and preservation of living body liquid, such as a microorganism and a cell. After diluting with the frost damage protective agent which can be received physiologically in such cryopreservation conventionally, freeze storage and the approach of saving are almost the case. However, freezing is cruel for a living thing and the survival rate is reduced in many cases for the thermal impact produced at freezing and a fusion process, or crystal generation. Therefore, it was anxious for development of the frost damage protective agent which raises the survival rate after cryopreservation more, and the cryopreservation approach.
[0004] In order to control the frost damage of a microorganism or a cell, the approach of diluting with the protective agent which can be received physiologically was examined, and it was found out that glycerol is effective (Polge, Nature (Nature), 164 volumes, 666 pages, 1949].). Development of the protective agent for controlling frost damage also after that is performed. For example, in lyophilized-products-izing of lactobacillus bifidus or lactic acid bacteria, the approach using lactulose (JP,52-151787,A), vitamin E (JP,53-5747,B), corn steep liquor (JP,58-104787,A), raw starch (JP,58-149675,A), and a cyclodextrin (JP,63-251080,A) as a protective agent added in order to raise a survival rate is learned. However, using this protective agent in large quantities, the matter of these approaches was expensive, and they had the trouble that palatability was not desirable etc. Moreover, when carrying out cryopreservation of the animal cell, the approach of needing addition of a blood serum in the freezing culture medium, or adding methyl cellulose, trehalose (Patent Publication Heisei No. 501112 [four to] official report) or trehalose, and gelatin (JP,5-7489,A) as a protective agent instead of a blood serum is also established. However, also in these approaches, improvement in a survival rate has been a technical problem.
[0005]
[Means for Solving the Problem] Then, this invention persons completed a header and this invention for the ability of extinction of a microorganism or a cell to be remarkably controlled at the time of cryopreservation or freeze drying by using an inulin mold fructan as a frost damage protective agent in a diluent, as a result of examining many things, in order to control extinction of the microorganism in a freezing process, or a cell.
[0006] After dipping a microorganism or a cell into the solution which contains an inulin mold fructan in the frost damage protective agent list for the microorganism characterized by this invention containing an inulin mold fructan as an active principle, or a cell, the cryopreservation approach of the microorganism characterized by making it freeze or freeze-dry or a cell is offered.
[0007] There are various kinds of things as a microorganism to which this invention is applied, for example, lactic acid bacteria, such as lactobacillus bifidus, such as Bifidobacterium address SENTESU, Bifidobacterium Inn Juan Tess, Bifidobacterium bifidum, Bifidobacterium longum, and Bifidobacterium breve, Streptococcus faecalis, and Lactobacillus acid philus, Escherichia coli like ESSHIERISHIA KORI K-12, a Bacillus subtilis like 168 shares of bacillus Subtilis mull BURUGU, and yeast like Saccharomyces SEREBISHIE can be mentioned.
[0008] Moreover, as an animal cell, the germ of Homo sapiens, a cow, a horse, a goat, the sheep, a rabbit, a hamster, a rat, and a mouse, a cancer cell, a T-cell leukemic cell, a hybridoma, fibrocyte, a blood vessel configuration cell, a bone marrow cell, a distributed islet cell, the cultured cell of fishes, etc. are mentioned. As a plant cell, cultured cells, such as a rice, wheat, a Madagascar periwinkle, corn, a sugarcane, tobacco, lavender, an apple, a ginseng, soybeans, a liverwort, a strawberry, a potato, a carnation, a pea, asparagus, MEKYABETSU, and a pear, a protoplast, a shoot apex, an adventious embryo, etc. can be mentioned.
[0009] After cultivating a microorganism by the culture medium of arbitration, a harvest is carried out according to centrifugal separation etc., the wet fungus body which washed by the suitable penetrant

remover is used, and a cell can use the cell cultivated by the approach of arbitration, such as shaking culture. The obtained microorganism or cell is used as suspension which added the buffer solution remaining as it is or little, and is made to suspend in the diluent containing the inulin mold fructan sterilized beforehand.

[0010] As an active principle, it is independent, or skim milk, dimethyl sulfoxide, a glycerol, grape sugar, cane sugar, a lactose, and vitamins can be combined suitably, and the component of a diluent can add them that it is simultaneous or additionally if needed including an inulin mold fructan as other components, and can be used.

[0011] The concentration of the inulin mold fructan used for this invention is preferably used in 1 - 40% of the weight of the range that what is necessary is just to use it by the concentration suitable for the microorganism to freeze or a cell. Moreover, the inulin mold fructan of this invention can be used as a part or all of a presentation of a frost damage protective agent. a with a polymerization degree of three or more to which the fructose has combined the inulin mold fructan with cane sugar in straight chain fructan -- it is -- usually -- polymerization degree 3-15 -- the thing of polymerization degree 3-6 is independent preferably, or it is used as two or more sorts of mixture.

[0012] Such inulin mold fructan or its mixture can be obtained extracting from chicory, an artichoke, etc., or by making the enzyme which has fructose transition capacity in cane sugar act. Although the following will be mentioned if a concrete inulin mold fructan is illustrated, this invention is not limited to these. For example, the inulin mold fructan obtained by the extract of an artichoke The thing (30 - 50 % of the weight) of polymerization degree 3-6, a with a polymerization degree of seven or more thing (20 - 50 % of the weight), The inulin mold fructan (trade name: MEIORIGO G; Meiji Seika Kaisha, Ltd. make) which have the sugar composition of a monosaccharide and 2 sugar (10 - 30 % of the weight), and the enzyme of the Aspergillus nigre origin is made to act on cane sugar, and is obtained The thing of polymerization degree 3-6 (55 - 60 % of the weight), It has the sugar composition of a monosaccharide and 2 sugar (40 - 45 % of the weight). Furthermore, the fructan mixture which uses the sugar of polymerization degree 3-6 as a principal component can be obtained by carrying out partial purification of the above-mentioned mixture using a column chromatography or the film. For example, by carrying out partial purification of above-mentioned MEIORIGO G using a column chromatography, the inulin mold fructan (trade name: MEIORIGOP) which contains the thing of polymerization degree 3-6 at 95% of the weight or more of a rate can be obtained, and the inulin mold fructan of polymerization degree 3-6 (70 % of the weight or more) can be obtained also from an artichoke extract. Furthermore, the inulin mold fructan 4 (nistose) which uses single polymerization degree as a principal component, for example, polymerization degree, and polymerization degree 5 (cell tosyl nistose) can be acquired by combining a column chromatography, crystallization, etc.

[0013] this invention frost damage protective agent for the microorganism characterized by containing an inulin mold fructan as an active principle or a cell shows the large improvement in a ratio (survival rate) which a microorganism and a cell survive after cryopreservation compared with the known frost damage protective agent containing the active principle known conventionally, and can prevent effectively the extinction in a microorganism or the freezing process of a cell.

[0014]

[Example] Next, although the example of this invention is shown, this is instantiation to the last and this invention is not limited to this.

Anaerobic culture of the 37 degrees C (Bifidobacterium longum) of the example 1 lactobacillus bifidus was carried out by BL culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- an anaerobic phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation --

the harvest was carried out. The obtained wet fungus body was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) After making the diluent only containing 6.7 % of the weight (inulin mold fructan of polymerization degree 4) of nistose suspend the obtained wet fungus body in homogeneity, it was made to freeze at -25 degrees C, and freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. Consequently, the numbers of micro organisms of a cryopreservation bacillus were 2.08x108 / ml, and the survival rate was 41.6%.
[0015] (b) After making the diluent only containing 6.7 % of the weight (presentation: 52% of things of polymerization degree 3-10, 34% of with a polymerization degree of 11 or more things) of inulin mold fructans extracted from the artichoke suspend a wet fungus body in homogeneity, the same actuation as said (b) was performed. Consequently, the numbers of micro organisms of a cryopreservation bacillus were 1.25x108 / ml, and the survival rate was 25.5%.
(c) After making the diluent which contains only 6.7 % of the weight (mol concentration 0.1M) of cane sugars as an example of contrast suspend a wet fungus body in homogeneity, when the same actuation as said (b) was performed, the numbers of micro organisms of a cryopreservation bacillus were 6.30x107 / ml, and the survival rate was 12.9%.
[0016] Anaerobic culture of the 37 degrees C (Bifidobacterium address SENTESU) of the example 2 lactobacillus bifidus was carried out by BL culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- an anaerobic phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 4 minutes, and the following experiments were presented.
(b) It mixed by the weight ratio 1:1 to the diluent containing 3 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and 10 % of the weight of skim milk, and the obtained wet fungus body was adjusted to pH7.0 (5 convention sodium-hydroxide solution). Mixed liquor was poured distributively on the petri dish and it freeze-dried at -20 degrees C. The number of micro organisms after freeze drying was 1.18x1011/g, and the survival rate was 33.5%.
[0017] (b) After mixing the diluent and wet fungus body containing the MEIORIGO P(Meiji Seika [Kaisha, Ltd.] make [a trade name,] the presentation: 44.4% [of things of polymerization degree 3], 42.9% [of things of polymerization degree 4], 8.9% [of things of polymerization degree 5], 0.6% of things of polymerization degree 6) 3 % of the weight and 10 % of the weight of skim milk which is the mixture of an inulin mold fructan by the weight ratio 1:1, the same actuation as said (b) was performed. The number of micro organisms after freeze drying was 1.08x1011/g, and the survival rate was 30.6%.
(c) After mixing the diluent and wet fungus body containing 3 % of the weight (inulin mold fructan of polymerization degree 5) of cell tosyl nistose, and 10 % of the weight of skim milk by the weight ratio 1:1, when the same actuation as said (b) was performed, the number of micro organisms after freeze drying was 1.09x1011/g, and the survival rate was 30.8%.
(d) After mixing a wet fungus body by the weight ratio 1:1 to the diluent which contains 3 % of the weight of lactoses, and 10 % of the weight of skim milk as an example of contrast, the same actuation as said (b) was performed. Consequently, the number of micro organisms after freeze drying was 5.88x1010/g, and the survival rate was 16.7%.
[0018] 37 degrees C (ESSHIERISHIA KORI K-12) of example 3 Escherichia coli were cultivated by L broth for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- a phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained

wet fungus body was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) The obtained wet fungus body was suspended at homogeneity in the diluent only containing 10 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and it froze at -25 degrees C. Freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. Consequently, the survival rate of the number of micro organisms of a cryopreservation bacillus was 41.6% in 3.99x108 / ml.
[0019] (b) When the diluent only containing 10 % of the weight (inulin mold fructan of polymerization degree 3) of 1-kestose was made to suspend a wet fungus body in homogeneity and the same actuation as said (b) was performed, the numbers of micro organisms of a cryopreservation bacillus were 2.47x108 / ml, and the survival rate was 25.8%.
(c) When the diluent which contains only 10 % of the weight of trehaloses as an example of contrast was made to suspend a wet fungus body in homogeneity and the same actuation as said (b) was performed, the survival rate of the number of micro organisms of a cryopreservation bacillus was 20.2% in 1.86x106 / ml.
(d) The diluent which contains only 10 % of the weight of cane sugars as an example of contrast was made to suspend a wet fungus body in homogeneity, and the same actuation as said (b) was performed. Consequently, the survival rate of the cryopreservation bacillus was 18.3% in the number of micro organisms 1.77x108 / ml.
[0020] 37 degrees C (Lactobacillus acid philus) of example 4 lactic acid bacteria were cultivated by the ILS culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- a phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 2 minutes, and the following experiments were presented.
[0021] (b) The wet fungus body was suspended at homogeneity in the diluent only containing 6.7 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and it froze at -25 degrees C. Freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. The survival rate of the number of micro organisms of a cryopreservation bacillus was 47.6% in 4.0x108 / ml.
(b) The diluent which contains only 6.7 % of the weight of cane sugars as an example of contrast was made to suspend a wet fungus body in homogeneity, and the same actuation as said (b) was performed. Consequently, the survival rate of the number of micro organisms of a cryopreservation bacillus was 15.7% in 1.32x108 / ml.
[0022] After cultivating at 37 degrees C by the MEM culture medium which added fetal calf serum 10% using the Hela cell of the cancer cell origin as example 5 cultured cell, it processed with trypsin liquid 0.25%, and the cell was removed. This was put into the centrifuging tube, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The obtained cell was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about nistose (inulin mold fructan of polymerization degree 4), it agitated, and the culture medium for freezing was prepared. It enclosed with ampul, after having added and suspended the cell in the culture medium for freezing, putting in the state of coldness and warmth for 1 to 2 hours and making a frost damage protective agent permeate intracellular. This ampul was frozen at -80 degrees C, and was saved for nine days. It diluted with the growth medium about 10 to 20 times after fusion with the 37-degree C thermostat, centrifugal was carried out for 5 - 10 minutes

by 500 - 600rpm, and the cell was collected. The dissolved cell checked life and death using trypan blue dyeing, and as a result of computing a survival rate, it survived 93%.
[0023] (b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glycerol as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 65%.
(c) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glucose as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 62%.
[0024] Ham's which added fetal calf serum 10% using CHO-K1 cell of the Chinese hamster ovary cell origin as example 6 cultured cell After cultivating at 37 degrees C by F12 culture medium, it processed with trypsin liquid 0.25%, and the cell was removed. This was put into the centrifuging tube, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The obtained cell was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about nistose (inulin mold fructan of polymerization degree 4), it agitated, and the culture medium for freezing was prepared. It enclosed with ampul, after having added and suspended the cell in the culture medium for freezing, putting in the state of coldness and warmth for 1 to 2 hours and making a frost damage protective agent permeate intracellular. This ampul was frozen at -80 degrees C, and was saved for nine days. It diluted with the growth medium about 10 to 20 times after fusion with the 37-degree C thermostat, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The dissolved cell survived 88%, as a result of checking life and death using trypan blue dyeing and computing a survival rate.
[0025] (b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glycerol as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 61%.
(c) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glucose as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 48%.
(d) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about dimethyl sulfoxide as an example of contrast, it agitated, and the culture medium for freezing was prepared. The same actuation as said (b) was performed for this. Consequently, the survival rate after freezing / fusion was 78%.
[0026] The callus of the hypocotyl origin of example 7 ginseng was cultivated by the liquid medium of suitable arbitration, it processed in the 0.7M mannitol by meicelase P-1 (a trade name, Meiji Seika Kaisha, Ltd. make), filtration, centrifugal, and washing of were done, and protoplast suspension was obtained. The obtained protoplast was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) You could add to the above-mentioned culture medium, it agitated, filtration and sterilization of were done, and it considered as the culture medium for freezing so that it might become the 20 % of the weight of the last concentration about nistose (inulin mold fructan of a degree of polymerization 4) and might become the 10% of the last concentration about dimethyl sulfoxide. protoplast suspension -- a coldness-and-warmth condition -- this culture medium for freezing -- every [small quantity] -- it

added, agitating quietly. After putting for 30 minutes to about 1 hour and making a frost damage protective agent permeate in a protoplast, it froze at -196 degrees C among liquid nitrogen, and saved for nine days. It diluted with the liquid medium which contains the rapid fusion back with a 37-degree C thermostat, and contains 0.4M mannitol in ice, centrifugal was carried out, and the protoplast was collected. The dissolved protoplast checked life and death using EBANZU blue dyeing, and as a result of computing a survival rate, it survived 52%.

[0027] (b) You could add to the above-mentioned culture medium, it agitated, and filtration and sterilization of were done so that it might become the 20 % of the weight of the last concentration about a glucose and might become the 10% of the last concentration about dimethyl sulfoxide as an example of contrast, and it considered as the culture medium for freezing. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 42%.

(c) You could add to the above-mentioned culture medium, it agitated, and filtration and sterilization of were done so that it might become the 20 % of the weight of the last concentration about shoe cloth and might become the 10% of the last concentration about dimethyl sulfoxide as an example of contrast, and it considered as the culture medium for freezing. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 28%.

[0028]

[Effect of the Invention] If the frost damage protective agent of this invention is used, in case the cell of a microorganism, vegetation, and an animal will be frozen, a freezing failure can be controlled and the survival rate can be raised. Therefore, in fields, such as food stuff industry and a pharmaceutical industry, usefulness of this invention is high.

TECHNICAL FIELD

[Industrial Application] This invention relates to the frost damage protective agent and the cryopreservation approach of making an inulin mold fructan an active principle. Furthermore, the freezing failure encountered in it when freezing the cultured cell of microorganisms, such as lactobacillus bifidus, lactic acid bacteria, and Escherichia coli, or vegetation, and an animal in a detail is controlled, and it is related with the frost damage protective agent and the cryopreservation approach of raising a survival rate.

EFFECT OF THE INVENTION

[Effect of the Invention] If the frost damage protective agent of this invention is used, in case the cell of a microorganism, vegetation, and an animal will be frozen, a freezing failure can be controlled and the survival rate can be raised. Therefore, in fields, such as food stuff industry and a pharmaceutical industry, usefulness of this invention is high.

TECHNICAL PROBLEM

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[0003] Generally, cryopreservation is performed for long term storage and preservation of living body liquid, such as a microorganism and a cell. After diluting with the frost damage protective agent which can be received physiologically in such cryopreservation conventionally, freeze storage and the approach of saving are almost the case. However, freezing is cruel for a living thing and the survival rate is reduced in many cases for the thermal impact produced at freezing and a fusion process, or crystal generation. Therefore, it was anxious for development of the frost damage protective agent which raises the survival rate after cryopreservation more, and the cryopreservation approach.

[0004] In order to control the frost damage of a microorganism or a cell, the approach of diluting with the protective agent which can be received physiologically was examined, and it was found out that glycerol is effective (Polge, Nature (Nature), 164 volumes, 666 pages, 1949].). Development of the protective agent for controlling frost damage also after that is performed. For example, in lyophilized-products-izing of lactobacillus bifidus or lactic acid bacteria, the approach using lactulose (JP,52-151787,A), vitamin E (JP,53-5747,B), corn steep liquor (JP,58-104787,A), raw starch (JP,58-149675,A), and a cyclodextrin (JP,63-251080,A) as a protective agent added in order to raise a survival rate is learned. However, using this protective agent in large quantities, the matter of these approaches was expensive, and they had the trouble that palatability was not desirable etc. Moreover, when carrying out cryopreservation of the animal cell, the approach of needing addition of a blood serum in the freezing culture medium, or adding methyl cellulose, trehalose (Patent Publication Heisei No. 501112 [four to] official report) or trehalose, and gelatin (JP,5-7489,A) as a protective agent instead of a blood serum is also established. However, also in these approaches, improvement in a survival rate has been a technical problem.

MEANS

[Means for Solving the Problem] Then, this invention persons completed a header and this invention for the ability of extinction of a microorganism or a cell to be remarkably controlled at the time of cryopreservation or freeze drying by using an inulin mold fructan as a frost damage protective agent in a diluent, as a result of examining many things, in order to control extinction of the microorganism in a freezing process, or a cell.

[0006] After dipping a microorganism or a cell into the solution which contains an inulin mold fructan in the frost damage protective agent list for the microorganism characterized by this invention containing an inulin mold fructan as an active principle, or a cell, the cryopreservation approach of the microorganism characterized by making it freeze or freeze-dry or a cell is offered.

[0007] There are various kinds of things as a microorganism to which this invention is applied, for example, lactic acid bacteria, such as lactobacillus bifidus, such as Bifidobacterium address SENTESU, Bifidobacterium Inn Juan Tess, Bifidobacterium bifidum, Bifidobacterium longum, and Bifidobacterium breve, Streptococcus faecalis, and Lactobacillus acid philus, Escherichia coli like ESSHIERISHIA KORI K-12, a Bacillus subtilis like 168 shares of bacillus Subtilis mull BURUGU,

and yeast like Saccharomyces SEREBISHIE can be mentioned.
[0008] Moreover, as an animal cell, the germ of Homo sapiens, a cow, a horse, a goat, the sheep, a rabbit, a hamster, a rat, and a mouse, a cancer cell, a T-cell leukemic cell, a hybridoma, fibrocyte, a blood vessel configuration cell, a bone marrow cell, a distributed islet cell, the cultured cell of fishes, etc. are mentioned. As a plant cell, cultured cells, such as a rice, wheat, a Madagascar periwinkle, corn, a sugarcane, tobacco, lavender, an apple, a ginseng, soybeans, a liverwort, a strawberry, a potato, a carnation, a pea, asparagus, MEKYABETSU, and a pear, a protoplast, a shoot apex, an adventious embryo, etc. can be mentioned.
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rate) which a microorganism and a cell survive after cryopreservation compared with the known frost damage protective agent containing the active principle known conventionally, and can prevent effectively the extinction in a microorganism or the freezing process of a cell.

EXAMPLE

[Example] Next, although the example of this invention is shown, this is instantiation to the last and this invention is not limited to this.
Anaerobic culture of the 37 degrees C (Bifidobacterium longum) of the example 1 lactobacillus bifidus was carried out by BL culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- an anaerobic phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) After making the diluent only containing 6.7 % of the weight (inulin mold fructan of polymerization degree 4) of nistose suspend the obtained wet fungus body in homogeneity, it was made to freeze at -25 degrees C, and freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. Consequently, the numbers of micro organisms of a cryopreservation bacillus were 2.08x108 / ml, and the survival rate was 41.6%.
[0015] (b) After making the diluent only containing 6.7 % of the weight (presentation: 52% of things of polymerization degree 3-10, 34% of with a polymerization degree of 11 or more things) of inulin mold fructans extracted from the artichoke suspend a wet fungus body in homogeneity, the same actuation as said (b) was performed. Consequently, the numbers of micro organisms of a cryopreservation bacillus were 1.25x108 / ml, and the survival rate was 25.5%.
(c) After making the diluent which contains only 6.7 % of the weight (mol concentration 0.1M) of cane sugars as an example of contrast suspend a wet fungus body in homogeneity, when the same actuation as said (b) was performed, the numbers of micro organisms of a cryopreservation bacillus were 6.30x107 / ml, and the survival rate was 12.9%.
[0016] Anaerobic culture of the 37 degrees C (Bifidobacterium address SENTESU) of the example 2 lactobacillus bifidus was carried out by BL culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- an anaerobic phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 4 minutes, and the following experiments were presented.
(b) It mixed by the weight ratio 1:1 to the diluent containing 3 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and 10 % of the weight of skim milk, and the obtained wet fungus body was adjusted to pH7.0 (5 convention sodium-hydroxide solution). Mixed liquor was poured distributively on the petri dish and it freeze-dried at -20 degrees C. The number of micro organisms after freeze drying was 1.18x1011/g, and the survival rate was 33.5%.
[0017] (b) After mixing the diluent and wet fungus body containing the MEIORIGO P(Meiji Seika [Kaisha, Ltd.] make [a trade name,] the presentation: 44.4% [of things of polymerization degree 3], 42.9% [of things of polymerization degree 4], 8.9% [of things of polymerization degree 5], 0.6% of things of polymerization degree 6) 3 % of the weight and 10 % of the weight of skim milk which is the mixture of an inulin mold fructan by the weight ratio 1:1, the same actuation as said (b) was

performed. The number of micro organisms after freeze drying was 1.08x1011/g, and the survival rate was 30.6%.
(c) After mixing the diluent and wet fungus body containing 3 % of the weight (inulin mold fructan of polymerization degree 5) of cell tosyl nistose, and 10 % of the weight of skim milk by the weight ratio 1:1, when the same actuation as said (b) was performed, the number of micro organisms after freeze drying was 1.09x1011/g, and the survival rate was 30.8%.
(d) After mixing a wet fungus body by the weight ratio 1:1 to the diluent which contains 3 % of the weight of lactoses, and 10 % of the weight of skim milk as an example of contrast, the same actuation as said (b) was performed. Consequently, the number of micro organisms after freeze drying was 5.88x1010/g, and the survival rate was 16.7%.
[0018] 37 degrees C (ESSHIERISHIA KORI K-12) of example 3 Escherichia coli were cultivated by L broth for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- a phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 3 minutes, and the following experiments were presented.
(b) The obtained wet fungus body was suspended at homogeneity in the diluent only containing 10 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and it froze at -25 degrees C. Freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. Consequently, the survival rate of the number of micro organisms of a cryopreservation bacillus was 41.6% in 3.99x108 / ml.
[0019] (b) When the diluent only containing 10 % of the weight (inulin mold fructan of polymerization degree 3) of 1-kestose was made to suspend a wet fungus body in homogeneity and the same actuation as said (b) was performed, the numbers of micro organisms of a cryopreservation bacillus were 2.47x108 / ml, and the survival rate was 25.8%.
(c) When the diluent which contains only 10 % of the weight of trehaloses as an example of contrast was made to suspend a wet fungus body in homogeneity and the same actuation as said (b) was performed, the survival rate of the number of micro organisms of a cryopreservation bacillus was 20.2% in 1.86x106 / ml.
(d) The diluent which contains only 10 % of the weight of cane sugars as an example of contrast was made to suspend a wet fungus body in homogeneity, and the same actuation as said (b) was performed. Consequently, the survival rate of the cryopreservation bacillus was 18.3% in the number of micro organisms 1.77x108 / ml.
[0020] 37 degrees C (Lactobacillus acid philus) of example 4 lactic acid bacteria were cultivated by the ILS culture medium for 24 hours, and centrifugal separation separated the fungus body from culture medium immediately after culture. the fungus body which carried out the harvest -- a phosphate buffer solution (pH7.0) -- washing -- this -- again -- centrifugal separation -- the harvest was carried out. The obtained wet fungus body was carried out for equivalent 2 minutes, and the following experiments were presented.
[0021] (b) The wet fungus body was suspended at homogeneity in the diluent only containing 6.7 % of the weight (inulin mold fructan of polymerization degree 4) of nistose, and it froze at -25 degrees C. Freezing and fusion (it is rapid fusion at freezing and 30 degrees C in -25 degrees C) were repeated 3 times after 24-hour preservation. The survival rate of the number of micro organisms of a cryopreservation bacillus was 47.6% in 4.0x108 / ml.
(b) The diluent which contains only 6.7 % of the weight of cane sugars as an example of contrast was made to suspend a wet fungus body in homogeneity, and the same actuation as said (b) was performed.

Consequently, the survival rate of the number of micro organisms of a cryopreservation bacillus was 15.7% in 1.32x108 / ml.

[0022] After cultivating at 37 degrees C by the MEM culture medium which added fetal calf serum 10% using the Hela cell of the cancer cell origin as example 5 cultured cell, it processed with trypsin liquid 0.25%, and the cell was removed. This was put into the centrifuging tube, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The obtained cell was carried out for equivalent 3 minutes, and the following experiments were presented.

(b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about nistose (inulin mold fructan of polymerization degree 4), it agitated, and the culture medium for freezing was prepared. It enclosed with ampul, after having added and suspended the cell in the culture medium for freezing, putting in the state of coldness and warmth for 1 to 2 hours and making a frost damage protective agent permeate intracellular. This ampul was frozen at -80 degrees C, and was saved for nine days. It diluted with the growth medium about 10 to 20 times after fusion with the 37-degree C thermostat, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The dissolved cell checked life and death using trypan blue dyeing, and as a result of computing a survival rate, it survived 93%.

[0023] (b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glycerol as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 65%.

(c) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glucose as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 62%.

[0024] Ham's which added fetal calf serum 10% using CHO-K1 cell of the Chinese hamster ovary cell origin as example 6 cultured cell After cultivating at 37 degrees C by F12 culture medium, it processed with trypsin liquid 0.25%, and the cell was removed. This was put into the centrifuging tube, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The obtained cell was carried out for equivalent 3 minutes, and the following experiments were presented.

(b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about nistose (inulin mold fructan of polymerization degree 4), it agitated, and the culture medium for freezing was prepared. It enclosed with ampul, after having added and suspended the cell in the culture medium for freezing, putting in the state of coldness and warmth for 1 to 2 hours and making a frost damage protective agent permeate intracellular. This ampul was frozen at -80 degrees C, and was saved for nine days. It diluted with the growth medium about 10 to 20 times after fusion with the 37-degree C thermostat, centrifugal was carried out for 5 - 10 minutes by 500 - 600rpm, and the cell was collected. The dissolved cell survived 88%, as a result of checking life and death using trypan blue dyeing and computing a survival rate.

[0025] (b) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glycerol as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 61%.

(c) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about a glucose as an example of contrast, it agitated, and the culture medium for freezing was prepared. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 48%.

(d) You could add to the growth medium which contains a blood serum so that it may become the 10 % of the weight of the last concentration about dimethyl sulfoxide as an example of contrast, it agitated, and the culture medium for freezing was prepared. The same actuation as said (b) was performed for this. Consequently, the survival rate after freezing / fusion was 78%.

[0026] The callus of the hypocotyl origin of example 7 ginseng was cultivated by the liquid medium of suitable arbitration, it processed in the 0.7M mannitol by meicelase P-1 (a trade name, Meiji Seika Kaisha, Ltd. make), filtration, centrifugal, and washing of were done, and protoplast suspension was obtained. The obtained protoplast was carried out for equivalent 3 minutes, and the following experiments were presented.

(b) You could add to the above-mentioned culture medium, it agitated, filtration and sterilization of were done, and it considered as the culture medium for freezing so that it might become the 20 % of the weight of the last concentration about nistose (inulin mold fructan of a degree of polymerization 4) and might become the 10% of the last concentration about dimethyl sulfoxide. protoplast suspension -- a coldness-and-warmth condition -- this culture medium for freezing -- every [small quantity] -- it added, agitating quietly. After putting for 30 minutes to about 1 hour and making a frost damage protective agent permeate in a protoplast, it froze at -196 degrees C among liquid nitrogen, and saved for nine days. It diluted with the liquid medium which contains the rapid fusion back with a 37-degree C thermostat, and contains 0.4M mannitol in ice, centrifugal was carried out, and the protoplast was collected. The dissolved protoplast checked life and death using EBANZU blue dyeing, and as a result of computing a survival rate, it survived 52%.

[0027] (b) You could add to the above-mentioned culture medium, it agitated, and filtration and sterilization of were done so that it might become the 20 % of the weight of the last concentration about a glucose and might become the 10% of the last concentration about dimethyl sulfoxide as an example of contrast, and it considered as the culture medium for freezing. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 42%.

(c) You could add to the above-mentioned culture medium, it agitated, and filtration and sterilization of were done so that it might become the 20 % of the weight of the last concentration about shoe cloth and might become the 10% of the last concentration about dimethyl sulfoxide as an example of contrast, and it considered as the culture medium for freezing. When the same actuation as said (b) was performed for this, the survival rate after freezing / fusion was 28%.